

基于假病毒的人乳头瘤病毒小鼠感染模型的建立和初步应用

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硕士学位论文

基于假病毒的人乳头瘤病毒小鼠感染模型
的建立和初步应用

Mouse Infection Model Based on Pseudovirus for Human
Papillomavirus and its Application

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摘要

人乳头瘤病毒（HPV）是引起宫颈癌和尖锐湿疣等疾病的主要因素，如何预防与治疗 HPV 引起的疾病已成为世界性的课题。HPV 预防性疫苗是目前公认的预防宫颈癌和尖锐湿疣发生的最有效手段。目前评价 HPV 疫苗保护性的主要标准是利用 HPV 假病毒检测免疫后的血清中是否含有高滴度的中和抗体，而产生的中和抗体是否具有可靠的保护性，这些数据在临床中较少。因此为了证明 HPV 疫苗是否能有效的预防人乳头瘤病毒的感染，需要合适的动物模型对疫苗的效果进行评价。

HPV 严格的种属特异性和组织特异性给 HPV 体外大量培养带来了极大的困难。研究发现通过质粒转染细胞获得的 HPV 假病毒与真病毒具有相似的形态结构和感染性，这些特征促使 HPV 假病毒替代真病毒构建动物感染模型。

本研究将经过密码子优化的 HPV16L1&L2 质粒和荧光素报告基因表达质粒通过 PEI 方法共转染 293FT 细胞，72 小时后收集裂解细胞，收获假病毒，利用活体成像仪对假病毒滴度进行测定。在实验过程中，发现质粒的质量及纯度直接影响着假病毒的滴度，因此为了保证实验中生产的假病毒可以满足检测需要，本研究通过摸索建立了质粒纯化及质粒质量检测的方法：利用 Sepharose 6FF 和 SOURCE 30Q 柱层析两步法纯化质粒，纯化后的质粒使用 Q sepharose TM Fast Flow 柱层析检测 ocDNA 和 ccDNA 的量和 G3000 检测纯度。

本研究又对 HPV 假病毒进行了深入的研究，通过 HPV16 双抗夹心系统检测出利用 293FT 细胞生产出的 HPV16 假病毒的浓度为 12 μ g/ml，Western-Blot 验证了假病毒中含有 HPV L1&L2 蛋白；为了研究 HPV 假病毒的结构，本文摸索了假病毒的纯化方法，确定了 Superose 6 prep grade 柱层析结合 Capto core 700 两步纯化假病毒的方法，可以达到较好的纯化效果，纯化后的假病毒在透射电镜视野下可以观察到大量直径为 60nm 的假病毒颗粒，为假病毒结构解析奠定了基础。

本研究将 HPV16L1&L2 和 Luc 报告基因质粒通过 PEI 共转染 293FT 细胞，获得包裹 Luc 报告基因的 HPV16 假病毒，利用活体成像仪检测 HPV16 假病毒滴度。通过注射醋酸甲羟孕酮使实验小鼠进入动情间期，壬苯醇醚-9（N-9）化学损伤其阴道，继而进行 HPV16 假病毒攻毒感染并用活体检测仪检测小鼠阴道中荧光素酶报告基因的表达情况。成功建立假病毒感染小鼠模型后，本研究又进行了假病毒滴度与最低感染剂量关系的摸索。为了将此模型应用于疫苗保护性检测，本研究又将 HPV16 疫苗分三种剂量免疫小鼠，免疫 6 周后利用 HPV16 假病毒小鼠感染模型评价 HPV16 疫苗的保护性，检测结果显示最低剂量（22ng）免疫的小鼠产生的中和抗体完全可以抵御 HPV16 PsV 的感染，表明了该假病毒感染模型具有较好的实际应用性。

综上所述，本研究成功地摸索出质粒纯化与质控及 HPV 假病毒颗粒纯化的方法，为稳定生产 HPV 假病毒和假病毒的低温电镜结构、L1&L2 相互作用研究奠定了基础；本研究建立的 HPV 假病毒小鼠感染模型，为 HPV 感染干预研究、致病、免疫逃逸、宫颈癌治疗药物及疫苗的研究奠定基础，并为鉴定 HPV 中和单抗和评价候选疫苗的免疫保护效果提供了有效手段。

关键词：人乳头瘤病毒 假病毒 小鼠感染模型 中和保护

Abstract

HPV is the main factor causing diseases such as cervical cancer and genital warts, while cervical cancer is the second most prevalent cancers of the worldwide. Therefore, how to prevent and treat the diseases caused by HPV have become a worldwide task. At present, HPV prophylactic vaccine has been recognized as the most effective methods to prevent cervical cancer and genital warts. Generally, people use the neutralizing antibodies in immune serum to evaluate the protection of HPV vaccines, which is tested by HPV pseudovirus. However, whether the neutralizing antibodies caused by immunization have reliable protection has not been confirmed clinically. In order to prove whether HPV vaccine can effectively prevent the infection of human papilloma virus, we need to establish appropriate animal model to evaluate the effects of the vaccine.

HPV has strict species specificity and tissue specificity, which has brought great difficulties for HPV culture in vitro. However, People found that HPV euvirus and pseudovirus got by plasmid transfecting cells had similar morphological structure and infection ability. These characteristics prompted that HPV pseudovirus can replace the true virus to build animal infection models.

In this study, we have used the plasmids of HPV16L1& L2 which have been disposed by codon optimization and pClucf as report gene to transfect 293FT cells by PEI methods. After 72 hours, we collected the cells by PBS, split them by lysis buffer and harvested the pseudovirus. End-point titer of PsV was detected on 293FT cells in 96-wells plates. After 48 hours of infection, D luciferine was added and luminescence was measured by Xenogen IVIS camera system. During the experiments, we had found that the concentration and purity of the plasmid directly affected the titers of PsV. Therefore, to ensure the PsV can meet the demand of our experiment, we had established the plasmid purification and plasmid quality testing methods, for example, using Sepharose 6FF and SOURCE 30Q column chromatography to purify the plasmids, using Q Sepharose TM Fast Flow column chromatography to detect the ocDNA and ccDNA. Otherwise,

G3000 column chromatography to detect the purity of our plasmids.

We also have done some in-depth research with the HPV pseudovirus. By HPV16 double-antibody sandwich ELISA, we found that the concentration of HPV pseudovirus produced by 293FT cells was 12 ug/ml. In addition, we also verify the pseudovirus containing HPV L1&L2 proteins by Western Blot. In order to research the structure of the HPV pseudovirus, we have also groped the purification methods of HPV pseudovirus, such as ultracentrifugation, S-1000, Sepharose CL-2B, Superose 6 prep grade and Capto core 700 column purification. Finally, purification method of the HPV pseudovirus was identified as this, Superose 6 prep grade combined with Capto core 700 column chromatography. This two-step purification method could achieve well purification effect. A large number of HPV pseudovirus particles can be observed with TEM and the diameter was about 60 nm.

HPV16 pseudovirus was generated in vitro by co-transfecting mammalian 293FT cells with three kinds of plasmids expressing the L1 and L2 capsid proteins of the papillomavirus of interest, along with Luc reporter plasmid. HPV16 pseudovirus was detected by living imaging detection system with a titer of 8.192×10^6 TRLU/ml. First day inject 100 μ l per mouse of a 30mg/ml solution of progesterone subcutaneously. The fourth day, lightly anesthetize the mouse in a box filled with isoflurane. Once anesthetized, place the mouse on its back with its nose resting in a nose cone filled with isoflurane. Collect 50 μ l of 4% Nonoxynol-9 into a positive displacement pipette. Insert the pipette tip ~1 cm into the vaginal tract. If you feel resistance, retract the tip 1~2 mm. After inserting the tip, occlude the vaginal entrance with forceps, instill the N-9 into the cervicovaginal tract, and slowly remove the tip. After 4~6 h, freshly prepare the pseudovirus preparation by diluting the desired amount of pseudovirus (no less than 2.5×10^7 IU) into CMC such that the final concentration of CMC is 2%. 36% Optiprep or sterile 1 \times PBS can be used to adjust the volumes to reach this concentration. After 48 h, using living imaging detection system detected the mice. The pseudovirus titer was 3.84×10^5 TRLU/ml. In order to compare different injection dose produced varying signals. Different immune dose was designed: 5 μ l, 10 μ l, 15 μ l, 20 μ l, 25 μ l. The test results showed that when more than 20 μ l HPV16 pseudovirus injected mouse, the signals was been detected.

In conclusion, this study successfully established the method of plasmid purification, quality control and the HPV pseudovirus particles purification. This achievement will stabilize the production of the HPV pseudovirus, promote the research of HPV pseudovirus structure using low temperature electron microscopic and L1&L2 interaction. Furthermore, the foundation of mouse infected models with HPV pseudovirus will promote the development of the research, such as, HPV infection, pathopoiesis, immune escape, cervical cancer drugs and vaccine, provide an effective methods for identifying the HPV neutralizing monoclonal antibodies and evaluating of the candidate vaccine immune protection .

Key words: Human papillomavirus HPV pseudovirus Mouse infected models Neutralizing protection

缩写词

Amp: Ampicillin, 氨苄青霉素
AUC: Analytical Ultracentrifugation, 分析型超速离心技术
APCs: Antigen Presenting Cells, 抗原呈递细胞
bp: base pair, 碱基对
BPV: Bovine Papillomavirus, 牛乳头瘤病毒
CDR: Complementarity Determining Region, 抗原互补决定区
CHT II: Calcium Hydroxyapatite II, II 型羟基磷灰石介质
CIN: Cervical Intraepithelial Neoplasia, 宫颈上皮内瘤样病变
CTL: Cytotoxic T Lymphocyte, 细胞毒 T 淋巴细胞
DC: Dendritic Cell, 树突状细胞
DLS: Dynamic Light Scattering, 动态光散射
DNA: Deoxyribonucleic Acid, 脱氧核糖核酸
ELISA: Enzyme-linked Immunosorbent Assay, 酶联免疫吸附测定
FDA: Food and Drug Administration, 美国食品及药品管理局
GAM-AP: 标记碱性磷酸酶的羊抗鼠抗体
GAM-HRP: 标记辣根过氧化物酶的羊抗鼠抗体
GFP: Green Fluorescent Protein, 绿色荧光蛋白
HPLC: High Performance Liquid Chromatography, 高效液相色谱
HPV: Human Papillomavirus, 人乳头瘤病毒
HRP: Horseradish Peroxidase, 辣根过氧化物酶
ICTV: International Committee on the Taxonomy of Viruses, 国际病毒学分类委员会
Kan: Kanamycin, 卡那霉素
KDa: kilo Daltons, 千道尔顿
NK: Natural Killer, 自然杀伤细胞
NMR: Nuclear Magnetic Resonance, 核磁共振
ORF: Open Reading Frame, 开放阅读框
PDB: Protein Data Bank, 蛋白质数据库
pRb: Retinoblastoma Tumor Suppressor Gene, 视网膜母细胞瘤抑制基因
RNA: Ribonucleic Acid, 核糖核酸
SAS: Solvent Accessible Surface, 溶剂可及化表面积
SPFF: Sp SepharoseTM Fast Flow, 阳离子交换层析介质
SV: Sedimentation Velocity, 沉降速度
TEM: Transmission Electron Microscopy, 透射电子显微镜
Th: Helper T lymphocyte, 辅助 T 淋巴细胞
URR: Upstream Regulatory Region, 上游调节区
VLPs: Virus-Like Particle(s), 类病毒颗粒

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前言

1 HPV 的分子生物学

1.1 HPV 研究概况

二十世纪后期,随着世界科学技术水平的快速发展,许多病毒与疾病之间的神秘面纱逐渐被人们揭开。在 1981 年 Gissmann, L.等人从人生殖器粘膜疣中分离出首个嗜黏膜型 HPV6,两年后他们又从相同的病变组织中分离得到 HPV11^[1,2]。从此,HPV 与生殖系统疾病被紧密联系起来;特别是德国科学家 Zur Hausen 在 1983 和 1984 两年间成功地从宫颈癌组织中分离出 HPV16 和 HPV18^[3,4],这一发现进一步阐明了 HPV 感染与宫颈癌的发生存在着密切的关系,并引起了前所未有的重视,吸引了众多研究者对 HPV 的预防与治疗展开研究,而 Zur Hausen 也由于这一重大研究成果获得了 2008 年医学与生理学诺贝尔奖。感染高危型 HPV 与宫颈癌发生的明确因果关系被确立后,HPV 的分子生物学被详细系统的研究,研究人员纷纷寻找预防 HPV 感染的药物及疫苗^[5]。在 2006 年,世界上第一支 HPV 预防性疫苗 Gardasil,已被美国食品药品监督管理局批准上市用于预防高危型 HPV 16、18 以及低危型 HPV6、11 的感染,它的上市标志着 HPV 研究又一里程碑式的突破。

1.2 HPV 结构及基因组

人乳头瘤病毒为乳头瘤病毒科(Papillomaviridae)乳头瘤病毒属,是无包膜的闭环双链 DNA 病毒。HPV 结构简单,仅由两种衣壳蛋白和核心单拷贝的基因组 DNA 构成。在电镜视野下 HPV 呈现为直径约 55nm 的球形病毒颗粒,利用低温电镜三维结构重建技术解析出该病毒颗粒为 T=7 的二十面体结构(如图 1)。

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